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The role of the glassy state in production and storage of freeze-dried starter cultures

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Abstract

The inactivation rates k of the model microorganism F19 (Chr. Hansen A/S, Hørsholm, Denmark) as well as the spin-spin relaxation time, T_2,were determined in the area around the glass transition temperature T_g. By keeping cell-sugar lyophilisates at defined temperatures and water activities, storage above and below T_g could be realized. In order to characterize the temperature dependence of T_2 and k, the results were fitted using the Williams-Landel-Ferry (WLF) and Arrhenius models. T_2 was found to be solely dependent on the temperature interval between T_g and the storage temperature, T_p. In case of inactivation kinetics this temperature interval could not fully explain the results. In contrast to molecular mobility, the microbial inactivation was supposed to be influenced additionally by the absolute storage temperature. This is due to fact that microbial inactivation is a highly complex process which is dependent on a number of physico-chemical reactions with some being more while some are less diffusion limited. In terms of inactivation kinetics and molecular mobility, T_g did not act as an absolute threshold but could divide the results into weakly and strongly temperature-dependent fractions. Within the freeze-drying process the glassy state of the sample matrix did not show a protective effect.

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Keywords: Freeze drying; Glass transition; Molecular Mobility; Inactivation kinetics; WLF;

1. Introduction

Starter cultures play an important role throughout the whole food industry. Thanks to their fermentative activity, many food products obtain their characteristic taste, aroma and structure. Moreover probiotic strains can add an additional health effect to the food products. A common way of stabilizing the cultures is drying [1]. However, during drying and subsequent storage, the culture suffers a significant loss in survival and activity. In order to minimize the detrimental effects, protectants (e. g. disaccharides) are added to the formulation prior to the drying step. Generally, this addition leads to a higher yield and improved storage stability. Due to the high complexity of the microorganisms, the underlying protection

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mechanisms determined from model proteins or liposomes cannot be simply applied to this system. One of the known protection mechanisms is the formation of a glassy matrix. Due to the restricted mobility within this matrix, the rates of detrimental physical, chemical and biochemical reactions are strongly depressed. Our aim was therefore to elucidate the role of the glassy state and molecular mobility in terms of microbial stabilization during freeze-drying and storage.

2. Material & Methods

2.1. Sample preparation and drying

Probiotic starter culture Lactobacillus. *paracasei* (F19) from Chr. Hansen A/S (Hørsholm, Denmark) was chosen as a model microorganism. The cells were provided as a frozen concentrate with a titer of around 10^{11}cfu/g (colony forming units). Cells were grown in a 4L batch fermentation process in Scharlau MRS broth (Barcelona, Spain) at 37°C, starting with an optical density (OD600) of about 0.3. The fermentation process was carried out without neutralization. After 10h, fermentation was stopped in the late exponential phase by cooling the cell suspension to 4°C. Cells were harvested by centrifugation (10 min, 4000g, 4 °C) to a final cell density of 10^{11}cfu/mL and washed twice with phosphate buffer saline (0.1M K_2HPO_4/KH_2PO_4 with 0.15M NaCl, pH 7.0). After washing, lactose (Merck, Darmstadt, Germany) was added to the cell suspension with 50 %(w/w) of the bio dry mass. This corresponds to an initial protectant concentration of 60 mg/mL. Exact aliquots of 1 mL of the sample suspension were transferred to 10 mL injection vials (Schärf Präzision Europa GmbH, Meiningen). The sample height was 2 mm. Samples from one fermentation were dried within one batch in a Delta 1-24 LSC freeze dryer (Martin Christ, Osterode, Germany). The shelves of the dryer were precooled to -20 or -40 °C depending on the chosen process. Samples were frozen to a final temperature of -60 °C by dipping vials into liquid nitrogen for 30 s. In case of storage test all samples were dried with the same three-step program (step 1: 14h 0.37 mbar/0 °C; step 2: 5.5h 0.12 mbar/15 °C; step 3: 3.5h 0.12 mbar/25 °C). In order to determine the influence of the glassy state on the drying result, process parameters were chosen according to Table 1. Due to the different process parameters the time to reach the glassy state of the sample matrix could be varied.

2.2. Determination of cell viability

Inactivation of the culture was quantified by determination of colony-forming units (cfu) before and directly after freeze drying and after certain time intervals during storage at defined conditions (see Table 2). The dry samples were rehydrated with sterile double-distilled water at 25 °C. Decimal dilutions of the samples were prepared with Ringer's solution. The number of viable cells was determined by the spread plate technique using MRS agar (1.5 %) and subsequent incubation at 37°C for 48 h under anaerobic conditions. Survival after freeze drying was defined as the percentage ratio of cells before and after drying. The survival after storage was defined as the percentage ratio of cells after drying and storage. Inactivation rates (k) were determined on basis of 4-6 weeks storage tests.

2.3. Lyophilization and microbalance

In order to investigate the influence of the glassy state on drying, different drying protocols with different residence times outside the glassy state were developed. Therefore, a freezing temperature of -20 °C were chosen. With this freezing condition the drying process starts in the non-glassy state whereupon the sample reaches the glassy state during drying due to further water removal. The transition of the sample into the glassy state, and thus the residence time in the non-glassy state, could be detected by introducing the microbalance CWS40 (Christ, Osterode, Germany) in the freeze-dryer according to the

method developed by Aschenbrenner et al. [2]. Due to this method different residence times in the non-glassy state could be determined.

2.4. Storage experiment

In order to evaluate storage stability, the dried samples were placed in glass containers with saturated solutions of different salts. Here, water activities aw ranging from 0.11 to 0.576 [3] and temperatures of 10, 25, and 37 °C were chosen. Equilibrium between samples and the surrounding atmosphere was reached after one week. As expected, due to water sorption, the initial glass transition temperatures (T_g) decreased during storage and finally reached an equilibrium T_g depending on the respective aw. Thus, the physical state of the sample, whether glassy or non-glassy, was dependent on the combination of aw and Temperature (Table 2).

2.5. Low resolution ^{1}H-NMR

Proton mobility of the samples was determined by means of low resolution ^{1}H-NMR with a minispec q20, Bruker Analytik GmbH (Rheinstetten, Germany). For measurements, an adequate number of samples were ground in a mortar, filled into a 10 mm NMR-tube and compressed. The radio-frequency (RF) used of the NMR-spectrometer was 20 MHz. The spin-spin relaxation time, T_2, was obtained by means of a combination of Free Induction Decay (FID) and Carr-Purcell-Meiboom-Gill sequence. In total 5000-pulses were applied with a pulse spacing of τ = 0.05 ms. A nonlinear fitting routine was applied to the resulting decay curve, which was best described by a sum of a Gaussian and an exponential component (equation (1)):

$$U(t) = a + f_S e^{\left(\frac{t}{2T_{2,1}}\right)^2} + f_L e^{\left(\frac{t}{T_{2,2}}\right)} \quad (1)$$

Where U(t) is the measured decay signal, f_S refers to the proton fraction of the solid component and f_L to the proton fraction of the liquid component. $T_{2,1}$ and $T_{2,2}$ are the relaxation times of the solid and mobile fractions. Within this context the term “solid” refers to molecules of reduced mobility such as biomass and solid protectants, whereas the liquid component represents the mobile protons from water and dissolved protectants. Based on these two fractions a total relaxation time T_2 was calculated as the weighted average.

2.6. Models

In order to determine the role of the glassy state as the stabilizing criterion, inactivation rates and proton mobilities below and above T_g were captured, compared to each other and fitted using the Arrhenius (Equation 2) and Williams-Landel-Ferry (WLF) models (Equation 3):

$$\eta = \eta_0 e^{-\frac{E_a}{RT}} \quad (2)$$

$$\log\frac{\eta}{\eta_S} = \frac{-C_1(T - T_S)}{C_2 + (T - T_S)} \quad (3)$$

where η_0 and η_s represent viscosities at a certain reference temperature T_S of the sample matrix which in case of the WLF-equation is T_g. The coefficients C_1 and C_2 have to be determined by the fitting procedure. Moreover, in case of restricted diffusion, the temperature dependency of reaction rates k can be described by these models due to the following relationship:

$$\frac{k_{T2}}{k_{T1}} \approx \frac{D_{T2}}{D_{T1}} \approx \frac{T2_{T2}}{T2_{T1}} \approx \left(\frac{\eta_{T1}}{\eta_{T2}}\right)^{C} \quad (4)$$

with reaction rate k, coefficient of diffusion D, spin-spin relaxation time T_2 and viscosity η. In this context, C represents a coupling factor. In case of perfect coupling of chemical and structural reactions C would become 1. Up to date, in all known cases k showed a weaker temperature dependence than η (C<1) [4, 5].

3. Results & Discussion

In order to investigate a potential protective effect of the glassy state within the drying process, three drying protocols were chosen (see Table 1) in order to realize different residence times outside the glassy state. The first parameter, T_{freeze}, gives the shelf temperature for the initial freezing step. The following three parameters relate to the drying process, where T_{shelf}, p_{drying} and $T_{sublimation}$ represent the shelf temperature, the pressure within the dryer and the corresponding sublimation temperature, respectively. The residence time outside the glassy state (t_g) could be determined by means of the microbalance. For PI the glassy state was reached by freezing to -40 °C, in case of PII and PIII after a certain time interval. X-ray diffraction measurements revealed that crystallization of lactose did not occur. The survival rates of the freeze-dried microorganisms, immediately after drying and after storage at defined conditions (a_w=0,33; T=25 °C), are shown in Figure 1.

Table 1. Overview of the applied drying protocols with freezing temperature T_{freeze}, shelf temperature T_{shelf}, chamber pressure p_{drying}, theoretical sublimation temperature $T_{sublimation}$ and time to reach the glassy state t_g

Protocol	T_{freeze} (°C)	T_{shelf} (°C)	P_{drying} (mbar)	$T_{sublimation}$ (°C)	t_g (h)
I	-40	-20	0.12	-40	0
II	-20	0	1.03	-20	2.9
III	-20	+20	0.12	-40	2.0

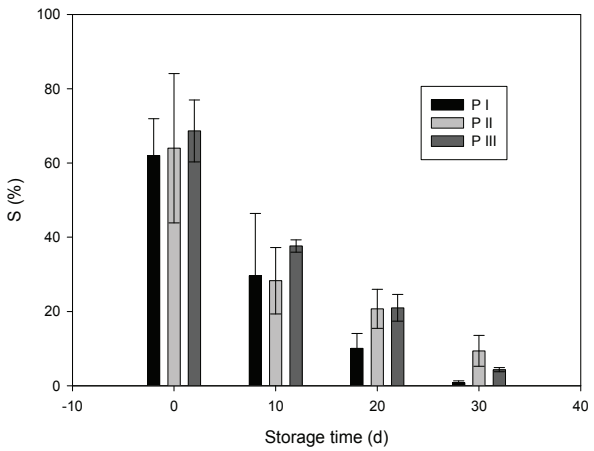

Fig. 1. Detected survival rates, S, of model microorganism dependent on process conditions, directly after drying (d=0) and after storage at a_w 0.33 and 25 °C. For drying protocol PI the sample stayed within the glassy state throughout the whole drying process. In case of PII and PIII the sample entered the glassy state after 2.9 and 2 h.

Immediately after drying the survival rates (S) are independent of the chosen drying protocol. Thus, a protective effect of the glassy matrix during drying cannot be recognized. However, in the case of storage stability, significant differences were visible. Here, the samples temporarily dried outside the glassy state (PI, PII) showed even higher storage stability. A similar effect was reported by Schersch et al. [6]. A possible explanation for these improved stabilities could be a densification of the protective matrix due to viscous flow during drying. In this context, further investigations will be necessary. In order to characterize the temperature dependence of the inactivation kinetics for F19, samples were stored at defined conditions (a_w, T) above or below the glass transition temperature T_g. As mentioned by Higl et al. [7], the microbial inactivation during storage could be described as 1st order kinetics. With increases in the temperature and/or aw, all samples showed increased inactivation rates. Table 2 gives an overview of all possible parameter conditions and the corresponding physical state of the sample (glassy/non-glassy).

Table 2. Overview of the chosen storage conditions water activity aw and temperature T_p. The term "glass" indicates that the sample is in the glassy state. In case of an empty cell the sample is outside the glassy state.

a_w (-) / T_p (°C)	0.11	0.23	0.33	0.43	0.58
10	Glass	Glass	Glass		
25	Glass	Glass			
37	Glass	Glass			

For all storage conditions the equilibrium, T_g was determined by DSC. Therefore, inactivation rates k as well as the spin-spin relaxation time T_2 could be plotted against the temperature interval T_p-T_g, where T_p represents the product temperature during storage and T_g the equilibrium glass transition temperature. In case of negative values (T_p-T_g<0), the sample matrix exists in the glassy state, whereas positive values (T_p-T_g>0) indicate a sample state outside the glassy state, the so-called rubbery state. As can be seen in Figure 2 the k-values for 10, 25 and 37 °C significantly deviate from each other. In case of the 10 °C storage no influence of the glassy state can be observed. Only for the highest aw value (highest value for T_p-T_g) a slight increase in k was observed. The samples stored at 25 °C show k values close to zero for storage within the glassy state. In the vicinity of T_g a gradual increase can be observed, followed by a slight linear increase. In contrast, the 37 °C samples show an exponential increase of k-values which already starts well below T_g. Thus, only in the medium temperature range (25 °C), T_g seems to have a threshold-like character. In general, the T_g of the sample matrix does not seem to be an absolute threshold and thus inactivation cannot be completely avoided. Nevertheless, in case of 37 °C, the storage inactivation of the model microorganism was significantly reduced within the glassy state. Therefore, a distinct protective effect can be reported. The fact that the inactivation rates cannot be described by one master curve is reason to believe that not only the distance to the glass transition temperature plays a role but also the absolute storage temperature. In order to characterize the temperature dependence of inactivation around the glassy state, the Arrhenius (Equation 2) and WLF (Equation 3) models were applied.

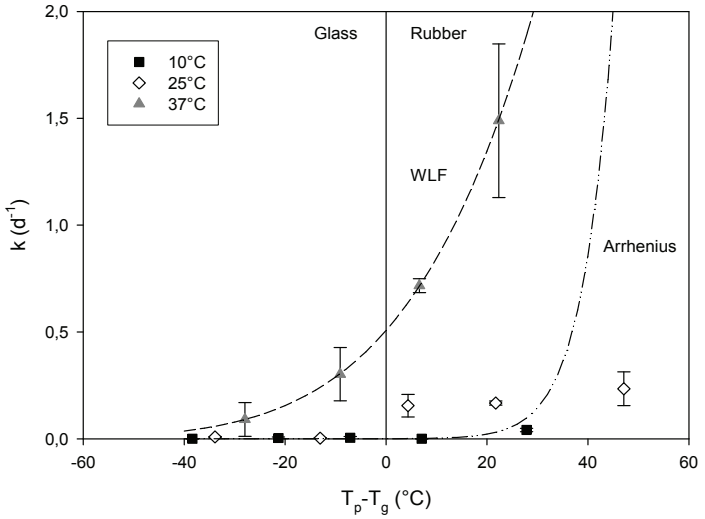

Fig. 2. Inactivation rates as a function of T_p-T_g. For the range of negative x-values the sample is in the glassy state, in case of positive values outside. The WLF and Arrhenius curve fitting results for the 37 °C storage are shown.

In case of the Arrhenius model the activation energy (E_a) was determined by means of an Arrhenius plot. In case of all aw conditions E_a reached values in the range of 120-160 kJ/mol. The curve fitting with the Arrhenius model (Figure 2) was accomplished on the basis of an average E_a of 143 kJ/mol. The WLF curve fitting was undertaken by adjusting the coefficients C_1 and C_2 using Sigma Plot software (Systat Software, Inc., San José, USA). In order to ensure readability of Figure 2 only the two fittings for storage temperature of 37 °C were introduced. All determined WLF coefficients can be found in Table 3.

Table 3. Fitting results for inactivation kinetics determined by WLF-equation with the inactivation rate at the glass transition temperature k_{Tg} and the universal coefficients C_1 and C_2.

T_p (°C)	k_{Tg} (T_p-T_g = 0)	C_1	C_2 (K)	R^2
10	0.004	28.2	1471.9	0.81
25	0.086	1.9	89.7	0.61
37	0.506	4.9	211.4	1.0

The estimated C_1 and C_2 values deviate significantly from the so-called universal coefficients (C_1 = 17.4 and C_2 = 51.6K) [8]. The regression curve on the basis of the universal coefficients (not shown) shows a much steeper course and thus stronger temperature dependence. However, these universal parameters relate to water-free polymer systems and are not necessarily suitable for complex food systems. For example, the formation of ice crystals in an ice-cream system could be described with C_1 and C_2 values of 20.4 and 154.8 K [9]. Thus, already a simple crystallization process showed distinct deviations from universal coefficients. Against this background the calculated values for a highly complex inactivation reaction seem to be plausible. Due to missing coefficients for microbial inactivation in the literature, comparison with the determined values was not yet possible. Generally, the good suitability of the WLF-model equation points to a diffusion-limited nature of the inactivation reaction, particularly in case of the 37 °C storage. The estimated E_a values, however, are in a range typical for oxidation and chemical reactions but well above diffusion limitation. However, due to a narrow

temperature range and the restricted number of measuring points the E_a values are limited on their reliability. Therefore, it is probable that the inactivation reaction is a very complex phenomenon, depending on a number of diverse reactions, some of them diffusion limited and some not. Besides the inactivation kinetics, the molecular mobility and its temperature dependence in the vicinity of the glass transition could be characterized by low resolution ^{1}H-NMR. Measurements were conducted for all storage conditions (Table 2). The relaxation behaviour of every sample was determined over a wide temperature range (0-60°C). Generally, low T_2-values indicate low proton mobility while high values show high proton mobility. Figure 3 shows the spin-spin relaxation time T_2 in dependence to T_p-T_g.

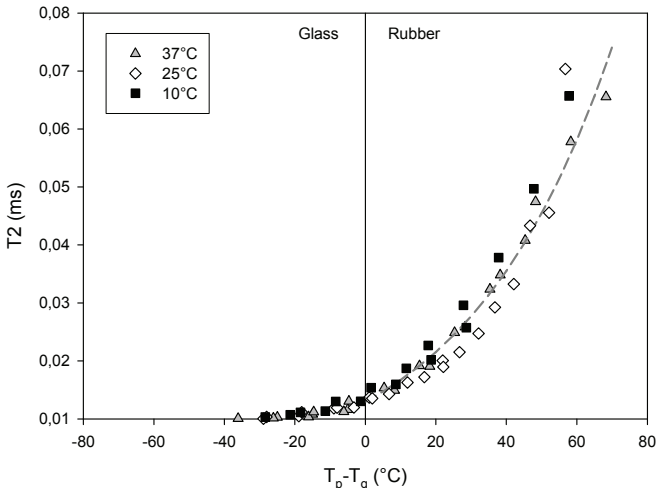

Fig. 3. Spin-spin relaxation times T_2 detected via ^{1}H-NMR for cell-sugar-samples. X-values in the negative temperature range indicate a glassy state of the sample, positive values indicate a non-glassy state.

The different data points represent all the combinations of measurement temperature and water activity. In contrast to inactivation kinetics, the T_2-values can be described by one master curve, independent of storage temperature. Thus, in case of molecular mobility only the temperature distance to the glassy state (T_p-T_g) plays a role. In case of all three storage temperatures, the relaxation times already start to increase at T_p-T_g = -20 °C. Obviously, at Tg the matrix already shows a slightly elevated mobility. Above T_g an exponential increase of the T_2-values is clearly visible. Thus, T_g separates the relaxation behavior into two regions of weak and strong temperature dependence. But as in the case of inactivation rates, T_g does not represent an absolute threshold. Curve fitting by means of WLF-model equation was applied and showed good agreement (see the dashed line in Figure 3). The calculated coefficients can be found in Table 4.

Table 4. Fitting results for molecular mobility at Tg determined by application of WLF-equation with spin-spin relaxation time at the glass transition T2Tg and the universal coefficients C1 and C2.

T_p (°C)	T_{2Tg} (ms) (T_p-T_g = 0)	C_1 (-)	C_2 (°C)	R^2
37	0.0129	26.7	2398.4	0.99

4. Conclusion

The application of the WLF-model equation revealed that the relaxation behavior of protons can be fully described, whereas inactivation kinetics can only be partly described as a function of T_p-T_g.

Therefore inactivation kinetics cannot solely be described by the molecular mobility. In both cases T_g does not represent an absolute threshold. Inactivation rates k (at 37 °C) as well as T2 times already start to increase in a temperature range 20 – 40 °C below T_g. In case of inactivation kinetics the relevance of T_g seems to be dependent on the absolute storage temperature. At low temperatures (10 °C), T_g does not play a role. In the middle temperature range (25 °C) a crossing of T_g goes ahead with a gradual increase of k. In case of the highest temperatures (37 °C) a slight increase of k below T_g turns into an exponential rise above Tg. The inactivation behavior at 37 °C was similar to the estimated relaxation behavior. Thus, in terms of inactivation kinetics the mobility in the vicinity of the glass transition suggested a bottle neck that limits the inactivation reaction to a certain degree. As already mentioned before, most likely the inactivation of the model microorganism is caused by a number of reactions being more or less diffusion limited. Thus, only a certain proportion of these reactions can be restricted through limited diffusion. The next step will be the characterization of these diffusion-limited reactions by modification of the storage conditions, replacement of protectants and further characterization of the protective matrix.

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References

[1] Santivarangkna C, Kulozik U, Foerst P. Alternative Drying Processes for the Industrial Preservation of Lactic Acid Starter Cultures. *Biotechnology Progress* 2007; **23**: 302–315.

[2] Aschenbrenner M, Kulozik U, Foerst P. 2011. In-situ determination of the physical state of biological samples during freeze-drying. *Drying Technology* 2011; **29**: 461–471.

[3] Greenspan L. Humidity fixed-points of binary saturated aqueous-solutions. *Journal of Research of the National Bureau of Standards – Section A – Physics and Chemistry* 1977; **81**: 89–96.

[4] Guo YS, Byrn SR., Zografi G. Effects of lyophilization on the physical characteristics and chemical stability of amorphous quinapril hydrochloride. *Pharmaceutical Research* 2000; **17**: 930–935.

[5] Shamblin SL, Hancock BC, Pikal MJ. Coupling between chemical reactivity and structural relaxation in pharmaceutical glasses. *Pharmaceutical Research* 2006; **23**: 2254–2268.

[6] Schersch K, Betz O, Garidel P, Muehlau S, Bassarab S, Winter G. Systematic Investigation of the Effect of Lyophilizate Collapse on Pharmaceutically Relevant Proteins I: Stability after Freeze-Drying. *Journal of Pharmaceutical Sciences* 2010; **99**: 2256–2278.

[7] Higl B, Kurtmann L, Carlsen CU, Ratjen J, Forst P, Skibsted LH, Kulozik U, Risbo J. Impact of water activity, temperature, and physical state on the storage stability of Lactobacillus paracasei ssp. paracasei freeze-dried in a lactose matrix. *Biotechnology Progress* 2007; **23**: 794–800.

[8] Williams ML, Landel RF, Ferry JD. The Temperature Dependence of Relaxation Mechanisms in Amorphous Polymers and other Glass-forming Liquids. *Journal of the American Chemical Society* 1955; **77**: 3701–3707.

[9] Hagiwara T, Hartel RW. Effect of sweetener, stabilizer, and storage temperature on ice re-crystallization in ice cream. *Journal of Dairy Science* 1996; **79**: 735–744.

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